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Subchronic exposures to fungal bioaerosols promotes allergic pulmonary inflammation in naïve mice

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Abstract

Background—Epidemiological surveys indicate that occupants of mold contaminated environments are at increased risk of respiratory symptoms. The immunological mechanisms associated with these responses require further characterization.

Objective—The aim of this study was to characterize the immunotoxicological outcomes following repeated inhalation of dry *Aspergillus fumigatus* spores aerosolized at concentrations potentially encountered in contaminated indoor environments.

Methods—*A. fumigatus* spores were delivered to the lungs of naïve BALB/cJ mice housed in a multi-animal nose-only chamber twice a week for a period of 13 weeks. Mice were evaluated at 24 and 48 hours post-exposure for histopathological changes in lung architecture, recruitment of specific immune cells to the airways, and serum antibody responses.

Result—Germinating *A. fumigatus* spores were observed in lungs along with persistent fungal debris in the perivascular regions of the lungs. Repeated exposures promoted pleocellular infiltration with concomitant epithelial mucus hypersecretion, goblet cell metaplasia, subepithelial fibrosis and enhanced airway hyperreactivity. Cellular infiltration in airways was predominated by $CD4^+$ T cells expressing the pro-allergic cytokine IL-13. Furthermore, our studies show that antifungal T cell responses (IFN-γ^+ or IL-$17A^+$) co-expressed IL-13, revealing a novel mechanism for the dysregulated immune response to inhaled fungi. Total IgE production was augmented in animals repeatedly exposed to *A. fumigatus*.

Conclusions & Clinical Relevance—Repeated inhalation of fungal aerosols resulted in significant pulmonary pathology mediated by dynamic shifts in specific immune populations and their cytokines. These studies provide novel insights into the immunological mechanisms and

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CONFLICT OF INTEREST

The authors have no competing financial interests to declare.

targets that govern the health outcomes that result from repeated inhalation of fungal bioaerosols in contaminated environments.

Keywords

fungi; subchronic; allergy; asthma; *Aspergillus fumigatus*; immunotoxicity

INTRODUCTION

Potential adverse health outcomes from fungal bioaerosols in contaminated buildings is of growing concern in the general population [1-3]. Consensus documents published by the Institute of Medicine (IOM) and the World Health Organization (WHO) have identified epidemiological associations between the presence of indoor mold contaminants and respiratory health effects [4, 5]. Recent updates to these documents have emphasized causal associations between fungal exposure and asthma exacerbations [6]. Fungal exposures can be significantly higher in water damaged indoor environments or in certain occupational environments such as agriculture and wood processing [7]. In mold contaminated indoor environments such as schools and offices, a concern for chronic exposure to molds exists [8, 9]. In such circumstances, individuals may be exposed to multiple fungal components such as spores, fragments, allergens, mycotoxins, and microbial volatile organic compounds (MVOCs) at varying rates depending on the extent of contamination.

Fungal spores or conidia of a respirable size gain access to deep pulmonary regions and are efficiently neutralized without excessive inflammatory damage [10]. However, in environments with excessive fungal contamination, exposures may be significantly higher resulting in robust inflammatory responses. Chronic exposure to fungal particles can exacerbate pre-existing respiratory distress and lead to rhinitis, asthma, and allergic diseases [11-14]. The impact of chronic exposures on human respiratory health and the immunopathological mechanisms that lead to allergic inflammation are poorly understood. Studies that model the normal exposure routes and the burden of fungi encountered in the environment are also lacking.

To date, most animal studies of fungal exposure have characterized pulmonary responses to liquid suspensions of spores or crude antigenic extracts in animals pre-sensitized to fungal antigens [15, 16]. These methods do not replicate an environmental exposure to fungal bioaerosols and do not consistently reproduce the pulmonary pathology associated with chronic exposures [17]. These methods instantaneously deliver a large bolus of spores that could potentially form agglomerates in the airways and influence the ensuing immune responses [18].

Recently, studies showed that exposure to dry fungal bioaerosols using a nose-only inhalation chamber resulted in significant expansion of the adaptive arm of the immune response in experimental animals [17, 19]. However, limitations in controlling the dose of inhaled fungal particles and the use of anesthesia may restrict an accurate assessment of the impact of natural fungal deposition in the lungs and limit the understanding of the underlying immunopathological mechanisms.

To address these methodological limitations, we developed an acoustical generator system (AGS) for delivering dry aerosolized fungal spores to mice housed in a multi-animal exposure chamber [20]. Aerosolized spores are delivered into a nose-only inhalation chamber, where mice inhale the spores. The system is equipped with a calibrated aerodynamic particle sizer that allows for real-time computation of particle size, concentration, estimated deposition, and adjustment of the lung deposition dose. In a recent report on short-term inhalational exposures of mice (4 weeks) to *A. fumigatus*, we observed significant recruitment of immune cells to the airways, dominated by a unique population of $CD8^{+}IL\text{-}17A^{+}$ (Tc17) T-cells [20]. In the present report, we expand those findings to address the pulmonary pathological responses associated with 13-week subchronic exposures and report the dynamic changes in recruitment of various immune cell populations associated with anti-fungal responses and allergic inflammation. The observations provide a better understanding of how the pulmonary response to molds may be regulated under long term exposure scenarios that typically represent a contaminated indoor environment.

METHODS

Animals, fungal cultures and exposures

Five week old female BALB/cJ mice were purchased from The Jackson Laboratory (Bar Harbor, ME) and housed in the NIOSH animal facility. All animal procedures were reviewed and approved by the NIOSH Animal Care and Use Committee (ACUC).

Aspergillus fumigatus B-5233 (gift from Dr. June Kwon-Chung, NIAID/Bethesda, MD) spores enriched on malt extract agar and after 7-14 days spores were harvested and resuspended in filter sterilized; endotoxin-free water (Sigma Aldrich, St. Louis, MO) and inoculated on autoclaved rice and cultured for 2 weeks.

Mice were separated into 3 exposure groups: 1) HEPA filtered air only (Control); 2) 1×10^4 *A. fumigatus* spores; or 3) 1×10^5 *A. fumigatus* spores. These doses represent the estimated deposited spores in lungs of animals for each exposure. Mice were exposed twice a week for a total of 13 weeks (total 26 exposures) using the Acoustical Generator System (AGS) as previously described [20]. The system is programmed to automatically shut off the spore supply when the desired dose of lung deposition has been achieved [20, 21]. Post-exposure exposure time points were selected to evaluate histopathological changes in the lung and for differential analysis of immune cells in murine tissues [20]. For airway hyperreactivity studies and serum antibody analysis, mice were sacrificed at varying time points post final exposures. Mice were euthanized *via* intraperitoneal injection of sodium pentobarbital solution (Sleepaway®, Fort Dodge, IA).

Histopathological assessment

Lung tissues were embedded in paraffin and sections were stained with hematoxylin and eosin (H&E), alcian blue/periodic acid-Schiff (AB/PAS) stain or Masson's trichrome stain. Fungal deposition in lungs was determined by Grocott's methenamine silver (GMS) staining. Total and germinating spores in GMS stained sections were quantified as previously described [20]. Spores > 3 μm in size were classified as swollen, suggesting germination.

Flow cytometry and differential analysis

Bronchoalveolar lavage fluid (BALF) and mediastinal lymph node (MLN) cell suspensions were prepared using previously described methods [20]. Cell suspensions for intracellular cytokine staining (ICS) were activated using Leukocyte activation cocktail with BD GolgiPlug™ (BD Biosciences). Prior to staining, Fc receptors were blocked by incubation in a cocktail containing unlabeled anti-CD16/CD32 (Fc block; BD Biosciences, San Diego, CA) and rat serum in FACS buffer (Dulbecco's phosphate buffered saline containing 5% fetal calf serum and 0.0005% sodium azide). For staining, fluorochromeconjugated antibodies such as PerCP-CD45, PE-Siglec-F, FITC-Ly-6G, V500-CD3ε, PE-CD45R/B220, AF700-CD4, APC-H7-CD8α, V500-CD45R/B220, BV421-CD25, APC-IL-5, PE-CF594-IFN-γ, AF647-IL-9 (all BD Biosciences, San Diego, CA), APC-CD11c, PE-IL-13, PerCP-EF710-IL-22 (all eBioscience, San Diego, CA), Pe-Cy7-IL-10, BV605-IL-17A (all BioLegend, San Diego, CA) were used per manufacturer's recommendations. Following surface staining, cell suspensions were fixed in BD CytoFix (BD Biosciences) prior to differential analysis of constituent populations or for ICS staining.

For differential analysis of BALF cell populations, leukocytes were first identified based on CD45 expression and then differentiated using a previously described strategy [22]. Neutrophils were identified as Ly-6G^{+} population. Ly-6G^{-} cells were further separated based on expression of Siglec-F and CD11c. Eosinophils were identified as CD11c^{low}Siglec-F^{high} populations. Alveolar macrophages were identified as CD11c^{high}Siglec-F^{+} populations on their autofluorescence profiles [23]. Lymphocytes were further differentiated based on expression of CD4 and CD8, while B cells were identified based on the expression of CD45R/B220. Within the lymphocyte subset in the BALF, cells with a surface phenotype CD4^{-}CD8^{-}B220^{-}CD25^{+} were defined as non-B non-T cell population (NBNT) and evaluated for cytokine expression profile. BALF (50,000 total events) and MLN (100,000 total events) cell populations were evaluated and differentiated using a BD LSRII flow cytometer system (BD Biosciences). Analysis of flow cytometry data was conducted using FlowJo v 7.6.5 software (Tree Star, Inc., Ashland, OR).

ELISA and Western blot analysis

Blood was collected at euthanasia in Microtainer serum separation tubes (BD Pharmingen, Franklin Lakes, NJ) and centrifuged to obtain serum. Total IgG_1 and IgG_{2a} in pooled sera were determined using standard methodologies described previously [24]. Serum IgE was estimated in pooled sera using a mouse IgE ELISA MAX™ kit as per manufacturer's instructions (BioLegend). *A. fumigatus* spore and hyphal extracts were generated using previously described methods [20, 25].

Airway hyperreactivity (AHR) Methodology

AHR assessment was conducted in BALB/cJ mice at 10 days post final exposure as previously described [26]. Briefly, mice were placed in Buxco whole body plethysmography chambers and baseline enhanced pause (Penh) values obtained. Mice were then sequentially challenged by nebulizing increasing concentrations (10, 25 and 50 mg/ml) of methacholine and Penh values determined.

Statistical analysis

Statistical differences between groups were evaluated by the test of analysis of variance (ANOVA) using SAS software. A Nonparametric Wilcoxon Rank Sum test was conducted to analyze total fungal counts and fungal germination data. For all graphs, $*p < 0.05$, $**p < 0.0001$ and n = 10 mice/group. Significance values are in comparison to control animals within the same time point, unless specifically noted.

RESULTS

Aerosolization and pulmonary deposition of A. fumigatus spores

The average aerodynamic diameter of aerosolized spores was 2.25 μm and spores were predominantly homogenous (**Figures 1A and 1B**). Mice from all exposure groups gained weight during the course of exposure interval (**Figure 1C**). Fungal deposition in murine lungs was multifocal and spores were readily observed in animals receiving 10^5 compared to 10^4 spores, with a higher spore burden observed at 24 hrs (**Figure 1D**). No fungal spores were observed in control animals. The presence of swollen spores and germ tube formation, particularly in animals exposed to the higher dose was suggestive that spores were in the process of germination (**Figures 1E and 1F**). Spores were primarily localized in the lung interstitium (**Figure 1E**), where staining was also observed for fungal debris. A pronounced staining was noted for fungal debris around the pulmonary blood vessels (**Figure 1G**). Although germination of *A. fumigatus* was observed, there was no evidence of extensive pulmonary colonization or invasive disease.

Pulmonary inflammation on repeated inhalation of spores

Inhalation of *A. fumigatus* spores resulted in a dose-dependent and multifocal peribronchial and perivascular inflammation characterized by pleocellular infiltration inclusive of mononuclear phagocytes, granulocytes and lymphocytes (H&E staining) (**Figure 2A**). This inflammatory response was most prominent in the bronchioles. Cellular infiltration was also prominent in the lung parenchyma surrounding the airways, and highlighted by significant alveolitis.

Exposure to fungal spores stimulated mucus production by the airways epithelial cells that was more pronounced at the 10^5 dose (**Figures 2A and 2B**). PAS staining demonstrated dose-dependent differences in goblet cell metaplasia in the bronchioles, and mice treated with the higher dose had larger numbers of cells with mucinogen granules (**Figure 2B**). Subepithelial fibrosis was assessed by trichrome staining and showed increased interstitial collagen deposition beneath the basement membrane of the airway epithelial cells (**Figure 2A**). Collagen deposition was prominent in regions of the lung that exhibited robust cellular infiltration.

Changes in the bronchial architecture were accompanied with minimal pulmonary arterial remodeling (**Supplementary Figure S1A**). Smaller blood vessels in close proximity to the bronchioles showed minimal narrowing of the lumina. Evidence of remodeling with collagen deposition was associated with increased cellular infiltration around blood vessels and was strongest in mice dosed with 10^5 spores. Remodeling that resulted in nearly

complete closure of the blood vessel lumen was rare and only observed in mice at the 10^5 dose (**Supplementary Figure S1B**). Blood vessels that demonstrated signs of remodeling typically showed perivascular deposition of fungal debris.

Cellular composition of airways indicate an allergic phenotype

Flow cytometric characterization of the cellular infiltrate in the airways demonstrated that subchronic inhalation of *A. fumigatus* spores increased recruitment of leukocytes to the airways (**Figure 3A**). A proportional reduction in alveolar macrophages (AMΦ) was observed in mice dosed with spores (**Figure 3B**). Infiltration by neutrophils and eosinophils increased significantly in a dose-dependent fashion and the former comprised the dominant innate cell population in the airways.

Repeated inhalation of fungal spores promoted the recruitment of adaptive immune cells to the inflamed airways (**Figure 3C**). The total number of $CD4^+$ (T_H) cells was significantly increased following the final exposure and by 48 hours formed the dominant cell population among all immune cells present in the airways. The total numbers of T_H cells recruited to airways was independent of the delivered dose in mice. The influx of $CD8^+$ T cells into the airways was minor. Although the recruitment of B cells significantly increased in mice dosed with 10^4 spores from 24 to 48 hrs, their recruitment to the airways was significantly lower compared to mice exposed to 10^5 spores.

Collectively these results demonstrate that repeated inhalation of *A. fumigatus* spores resulted in the formation of a mixed immune response in the airways; with significant dose-dependent differences in the recruitment of neutrophils, eosinophils and B cells. Other innate and adaptive responses did not significantly differ in animals exposed to different doses of fungal spores over a period of 13 weeks.

Recruitment of T cells with diverse cytokine expression profiles

Adaptive immune cells recruited to the airways secrete various mediators following inhalation of fungi and contribute to changes in pulmonary architecture and pathology. To further define the T-cell immune responses, intracellular cytokine expression profiles were examined using flow cytometry. A complex mixture of T_H1, T_H2, T_H17 and T_H22 cells was observed in the airways with significantly elevated numbers of $IL\text{-}13^+CD4^+$ T cells (T_H2) indicating a dominant allergic response (**Figure 3D and Supplementary Figure S2**). This robust expansion in the number of T_H cells expressing IL-13 demonstrated both, an increase in the mean fluorescence intensity (MFI) for IL-13 and dose-dependent differences that were significant at 48 hours (**Figure 3E**). $CD4^+$ T cells that expressed the pro-inflammatory cytokine IFN-γ (T_H1) were identified as the second largest T_H population. The recruitment of $CD4^+$ T cells expressing the pro-inflammatory cytokine IL-17A (T_H17) cells was significantly increased between doses. The T_H17 cells did not express IFN-γ (data not shown) and hence represent the regulated “classical” T_H17 subpopulation [27]. IL-22 expressing $CD4^+$ T cells (T_H22) were recruited to the airways and their numbers were comparable to T_H17 cells (**Figure 3D**). Furthermore, >95% of IL-22 expressing $CD4^+$ T cells did not co-express IL-17A (**Figure 3F**) and were thus identified as a T_H22 cell population. T_H22 cells have been previously reported to be important in anti-microbial host

defense, in limiting mucosal inflammation and in playing a role in tissue repair following repeated microbial insult [28, 29].

T_H cells expressing IFN-γ, IL-17A and IL-22 drive pulmonary anti-fungal responses by recruiting innate cells and promoting sequestration of inhaled fungal spores. We analyzed the expression of IL-13 in CD4 T cells expressing IFN-γ (**Figures 4A - 4C**) or IL-17A (**Figures 4D - 4F**) or IL-22 (**Figures 4G - 4I**). We observed that T_H1 (30-40%), T_H17 (25-35%) and T_H22 (10-20%) cells expressed the pro-allergenic T_H2 cytokine IL-13 in a dose-dependent fashion although dose-dependent differences were not significant. The mean fluorescence intensity for IL-13 expression in these subpopulations was significantly different between doses at 24 hours (T_H17 only) and 48 hours (for all subpopulations).

In the MLNs, B cells constituted the largest cell population followed by $CD4^+$ T cells (**Supplementary Figure S3A**). Expansion of both populations was greater in animals repeatedly dosed to 10^5 spores. Significant differences were demonstrated in the number of $CD8^+$ T cells in the MLNs. The cytokine profile of $CD4^+$ and $CD8^+$ T cells largely paralleled that of the subpopulations in the BALF. Among $CD4^+$ T cells, IL-13 expressing cells along with IL-9 expressing cells formed the largest populations followed by IL-22, IFN-γ, IL-10 and then IL-17A expressing populations (**Supplementary Figure S3B**), while most $CD8^+$ T cells expressed IFN-γ (**Supplementary Figure S3C**).

Collectively, these results show that IL-13 responses dominate the airways of animals that repeatedly inhaled fungi, and dose-dependent differences were not significant; although increased expression of IL-13 was observed in recruited T_H cells. Furthermore, the plasticity of the anti-fungal T cell responses also appeared to contribute towards the total IL-13 pool, thus identifying a novel mechanism driving the allergic responses.

Antibody responses to repeated inhalation of spores

Repeated inhalation of spores stimulated IgG and IgE antibody production in *A. fumigatus* exposed mice (**Figure 5**). A significant increase in total serum IgE levels was observed, which continued to increase 2 weeks after the completion of fungal exposures (**Figure 5A**). Serum IgE concentrations were also distinctly elevated in animals receiving the higher dose of the respirable fungal spores.

Elevated levels of IgG_1 and IgG_{2a} serum antibodies are associated with T_H2 and T_H1 responses respectively. An IgG_{2a}/IgG_1 ratio of >1 is supportive of the T_H1 type of response, while a ratio of <1 is indicative of a T_H2 type response. This ratio was consistently >1 at both time points in animals dosed with 10^4 spores (**Figure 5B**). In contrast, exposure of mice to 10^5 spores resulted in a ratio of <1 following, suggesting a shift to an allergic phenotype.

Inhalation of A. fumigatus results in enhanced AHR

In order to determine the impact of elevated allergic responses in the airways, we assessed airway hyperreactivity in mice at 10 days post final exposures (**Figure 5C**). Repeated inhalation of *A. fumigatus* by mice over a period of 13 weeks resulted in enhanced airway hyperresponsiveness to aerosolized methacholine with dose-dependent differences at 50

mg/ml methacholine after 10 days following the final exposure. All data points reflect values relative to baseline values determined with no methacholine challenge.

DISCUSSION

Mold contamination in damp indoor environments and the exacerbation of adverse respiratory health effects has gained significant attention in recent times. To date, few murine exposure models have replicated mold exposures that would be encountered in the environment. Furthermore, the broad diversity of various molds poses significant challenges in evaluating toxicity. To overcome these limitations, NIOSH has developed an exposure system that allows the study of the health effects resulting from the inhalation of fungal bioaerosols.

Short term *A. fumigatus* spore inhalation exposures (4 weeks) were found to induce an acute inflammation highlighted by a notable $CD8^+IL\text{-}17A^+$ (Tc17) response that correlated with *in vivo* spore germination [20]. In the present study, subchronic exposures (13 weeks) in naïve animals skewed the immune response toward an allergic phenotype resulting in significant expansion of diverse subpopulations of $CD4^+$ T cells and Tc17 cells no longer detectable. Our results additionally demonstrate that a small proportion of inhaled spores were in the process of germination in the lungs. During in vitro assessments, it has been shown that germinated fungal spores release greater quantities of allergens than un-germinated fungal spores [30]. This process may provide persistent antigenic stimulation in situ, influence the dynamic nature of pathogen recognition and promote the expansion of adaptive immune responses in the pulmonary environment.

Chronic inflammation following exposure to *A. fumigatus* spores for 13 weeks was characterized by the influx of a complex mixture of cells that expressed cytokines associated with T_H1, T_H2, T_H17 and T_H22 responses. Most $CD4^+$ T cells expressed IL-13, the hallmark cytokine of the T_H2 response and a central mediator of allergic inflammation and airway diseases. Allergic disease features consistent with IL-13 effector functions [31], such as increased eosinophilia, isotype switching in B cells, IgE production, mucus secretion, goblet cell metaplasia, pulmonary tissue remodeling, enhanced airway hyperreactivity and subepithelial fibrosis were observed. Fibrosis resulting from inefficient clearance of a pathogen and its related constituents causes persistent and ineffective chronic inflammation and injury [32]. Persistence of intact fungi and fungal debris post exposures may be a contributing factor in the current study. The allergic response is also likely to be supported, in part, by T cells expressing the cytokines, IL-5 and IL-9.

Recent reports describe plasticity among T_H17 and transitional stage T_H1 effector populations in secreting IL-13 in mice and humans in response to foreign antigens [33-36]. This plasticity is owed to alternative transcription factor usage by antigen presenting cells that promote re-programming of T_H1 and T_H17 cells to secrete T_H2 cytokines. Using intracellular cytokine staining to characterize co-expression of cytokines by individual cells, our studies show that an appreciable fraction of T_H1 and T_H17 cells produce IL-13 *in vivo* following repeated exposures to *A. fumigatus* spores. This is a critical finding as it demonstrates that a significant fraction of T cells that drive anti-fungal responses, also

contribute to the allergic outcomes. Identifying specific antigen presenting subpopulations driving the polarization signals resulting in mixed anti-fungal/allergy phenotype in these T cells could provide insight into how allergic diseases to fungi develop.

Collectively, our studies used significantly lower doses than previous animal models of fungal exposures [10, 37] and showed that repeated inhalation of fungal aerosols can result in dysregulation of immunological mediators impacting the pulmonary architecture and function. This may be due to the adaptive antifungal responses that contribute towards the development of allergic disease. However, ILCs and more specifically inflammatory ILC2s may in part contribute to the overall dysregulation [16, 38, 39]. These studies emphasize the role of pulmonary innate recognition of inhaled fungal spores in providing composite signals for promoting fungal clearance and allergic responses. Furthermore, fungal germination was identified in the lungs of exposed mice and highlight an additional source of antigenic stimulation that could contribute towards development of allergic inflammation. The results of this study suggest that personal exposure to low doses of fungal bioaerosols for long durations can result in the development of allergic responses involving moderate airway inflammation.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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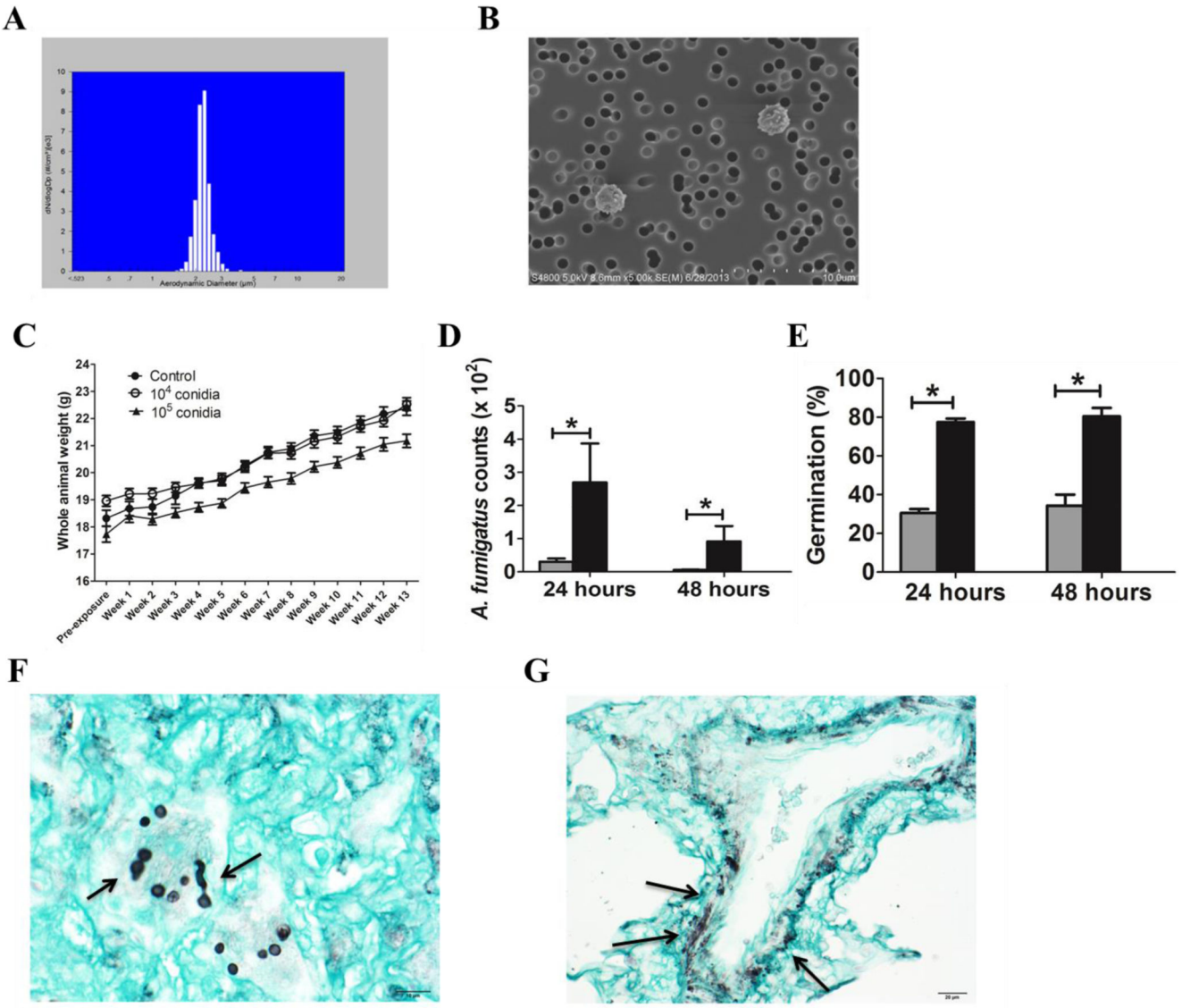

Figure 1. Spore dispersal, animal weights and spore germination *in vivo*

A. Graph representing mean size of particles aerosolized during exposure. All aerosolized spores were under 3 μm in size. **B**. Scanning electron microscope (SEM) image of aerosolized *Aspergillus fumigatus* spores deposited on a polycarbonate filter. **C.** Effect of repeated exposures to *A. fumigatus* spores on whole animal body weight. Control (●), 10^4 *A. fumigatus* spores (○) and10^5 *A. fumigatus* spores (▲) (n = 28-30 mice per group). **D.** Total *A. fumigatus* spore counts post exposure. Exposure groups - 10^4 spores (grey bars) and 10^5 spores (black bars) (n=2-3). **E.** Percentage of *A. fumigatus* spores germinating post exposure (n=2-3). **F.** Representative micrograph of GMS stained section from animal dosed with 10^5 spores at 24 hr post exposure (n=2-3). Black arrows point to germinating spores. Magnification: 100X objective and bar size = 20 μm. **G.** Micrograph of GMS stained section from animal dosed with 10^5 spores, 24 hrs post exposure showing perivascular deposition of fungal debris (black arrows) (n=2-3). Magnification: 60X objective and bar size = 20 μm.

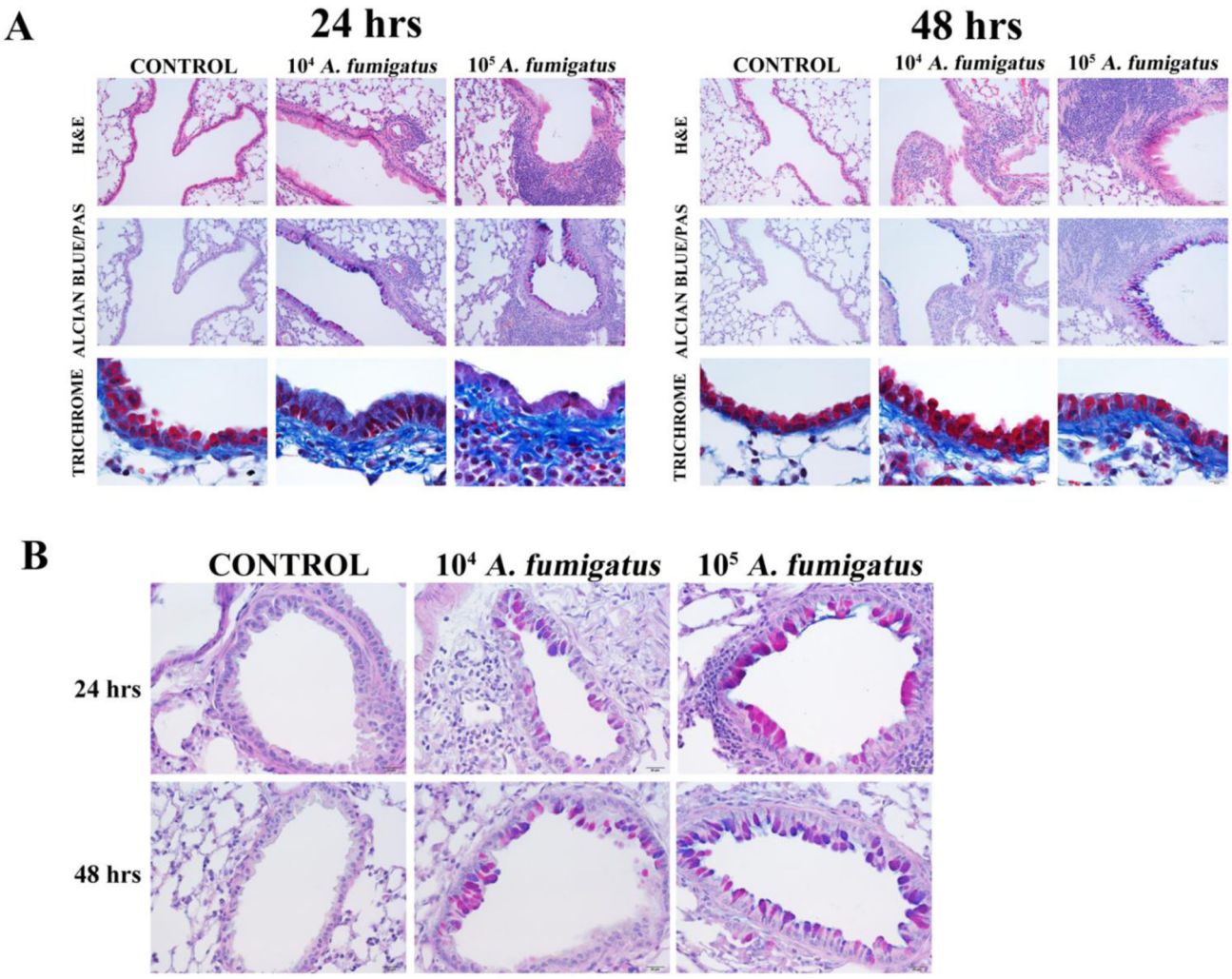

Figure 2. Repeated inhalation of fungal spores causes allergic pulmonary inflammation and changes in pulmonary airway architecture

A. Representative stained lung tissue sections from animals at 24 and 48 hrs post final exposure. Magnification: 20X objective for H&E and AB/PAS stained sections and 100X for Trichrome stained sections (n=2-3). **B.** Representative AB/PAS stained lung sections generated from right lungs of animals, excised at 24 and 48 hrs post final exposure (n=2-3). Magnification: 40X objective. H&E – hematoxylin and eosin. AB/PAS – alcian blue/ periodic acid-Schiff. Bar size – 50 μm (H&E and AB/PAS staining) and 20 μm (Trichrome staining).

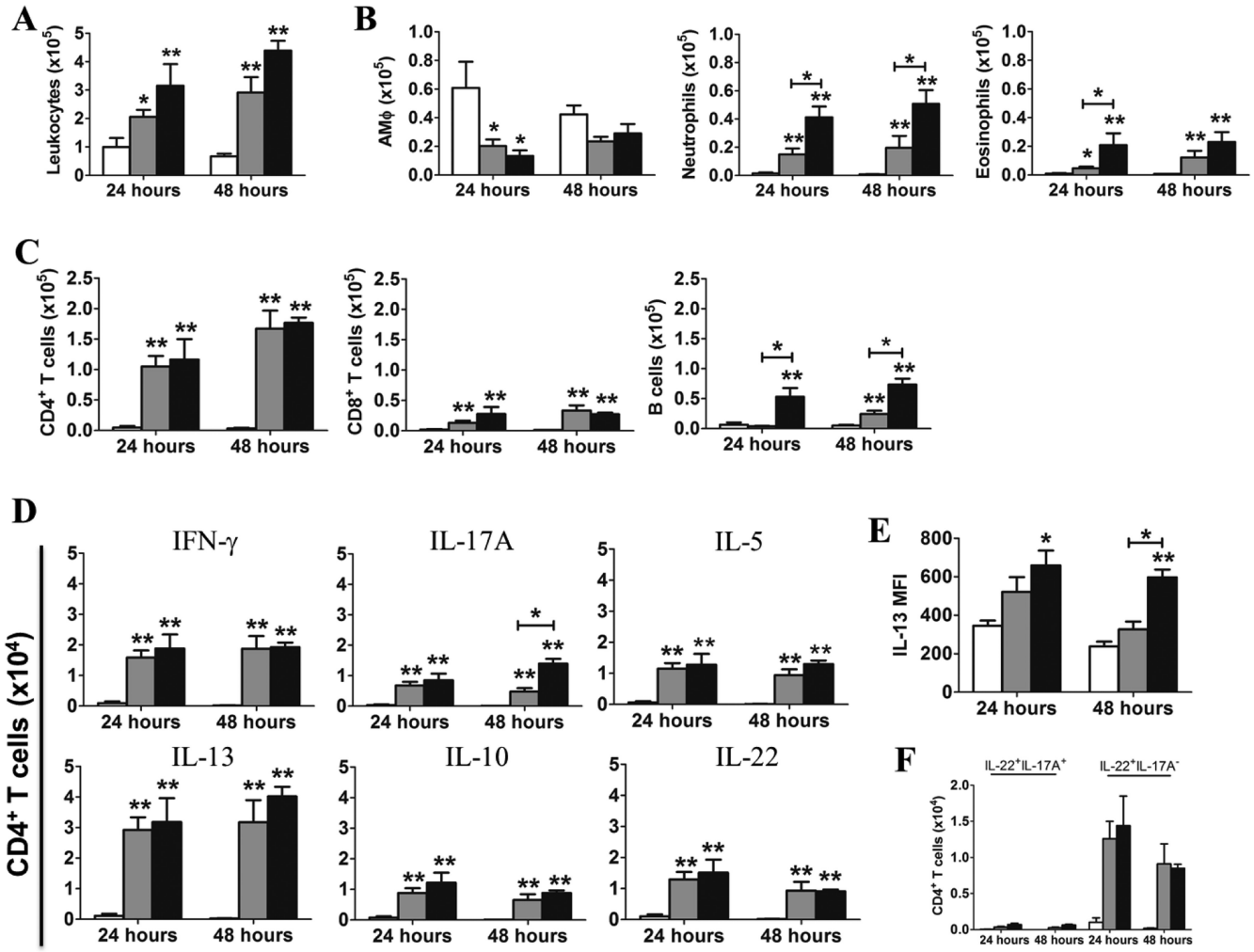

Figure 3. Exposure to dry aerosols of *A. fumigatus* spores promotes infiltration by immune cells in the airways

A. Recruitment of leukocytes, **B**. alveolar macrophages (AMΦ), neutrophils and eosinophils and **C.** adaptive immune cells to the airways. **D.** Cytokine expression profile of $CD4^+$ T cells (T_H) in the airways. **E.** MFI for $CD4^+IL\text{-}13^+$ T cell population. **F**. Distribution of $IL\text{-}17A^+$ and $IL\text{-}17A^-$ subpopulations of T_H22 cells in the murine airways. Exposure groups - (white bars) Control, (grey bars) 10^4 spores, (black bars) 10^5 spores. MFI – mean fluorescence intensity. For all graphs (n=8-10).

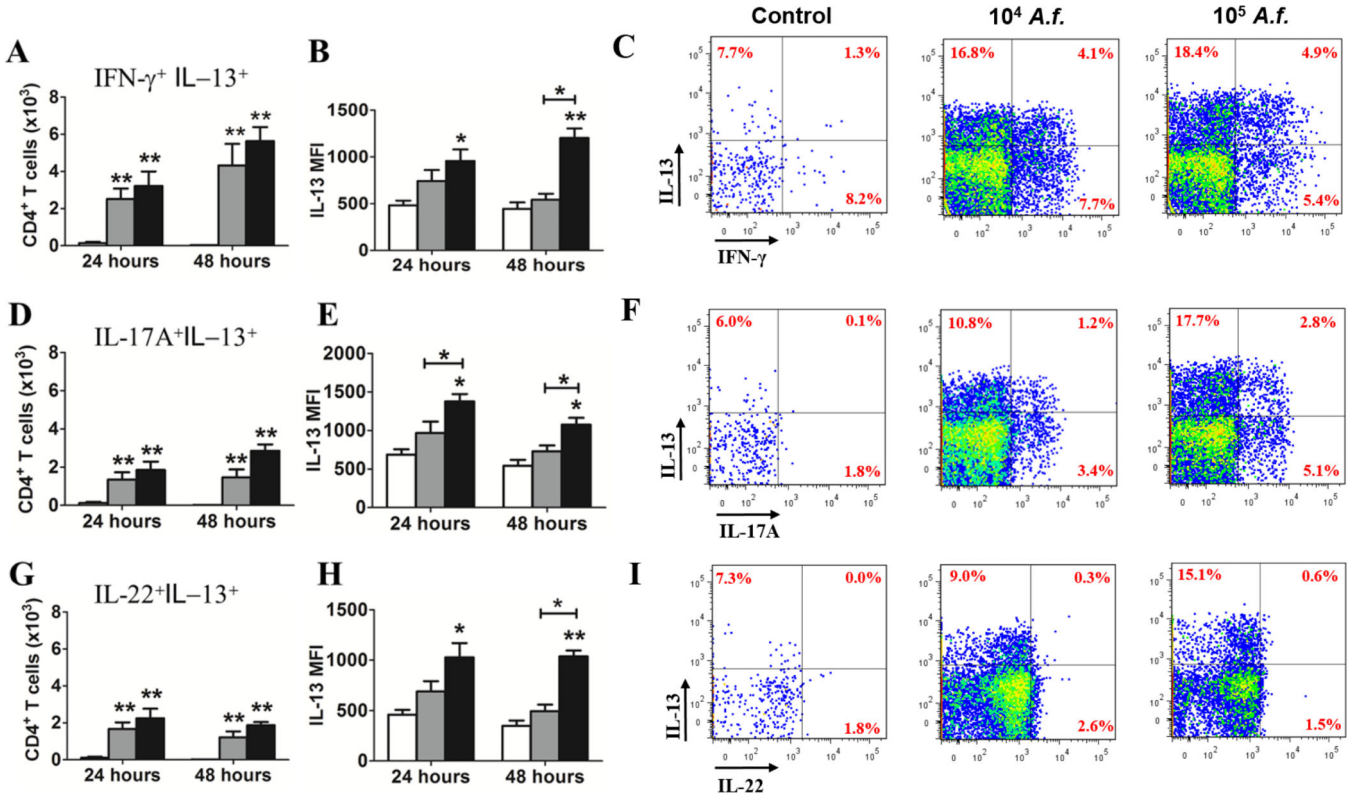

Figure 4. Accumulation of bi-functional CD4$^+$ T cells in airways of mice inhaling *A. fumigatus*

Co-expression profile of cytokines on gated CD4$^+$ T lymphocytes, **A-C.** IFN-γ/IL-13, **D-F.** IL-17A/IL-13 and **G-I.** IL-22/IL-13. Exposure groups - (white bars) Control, (grey bars) 10^4 spores, (black bars) 10^5 spores. MFI – mean fluorescence intensity. For all graphs (n=8-10).

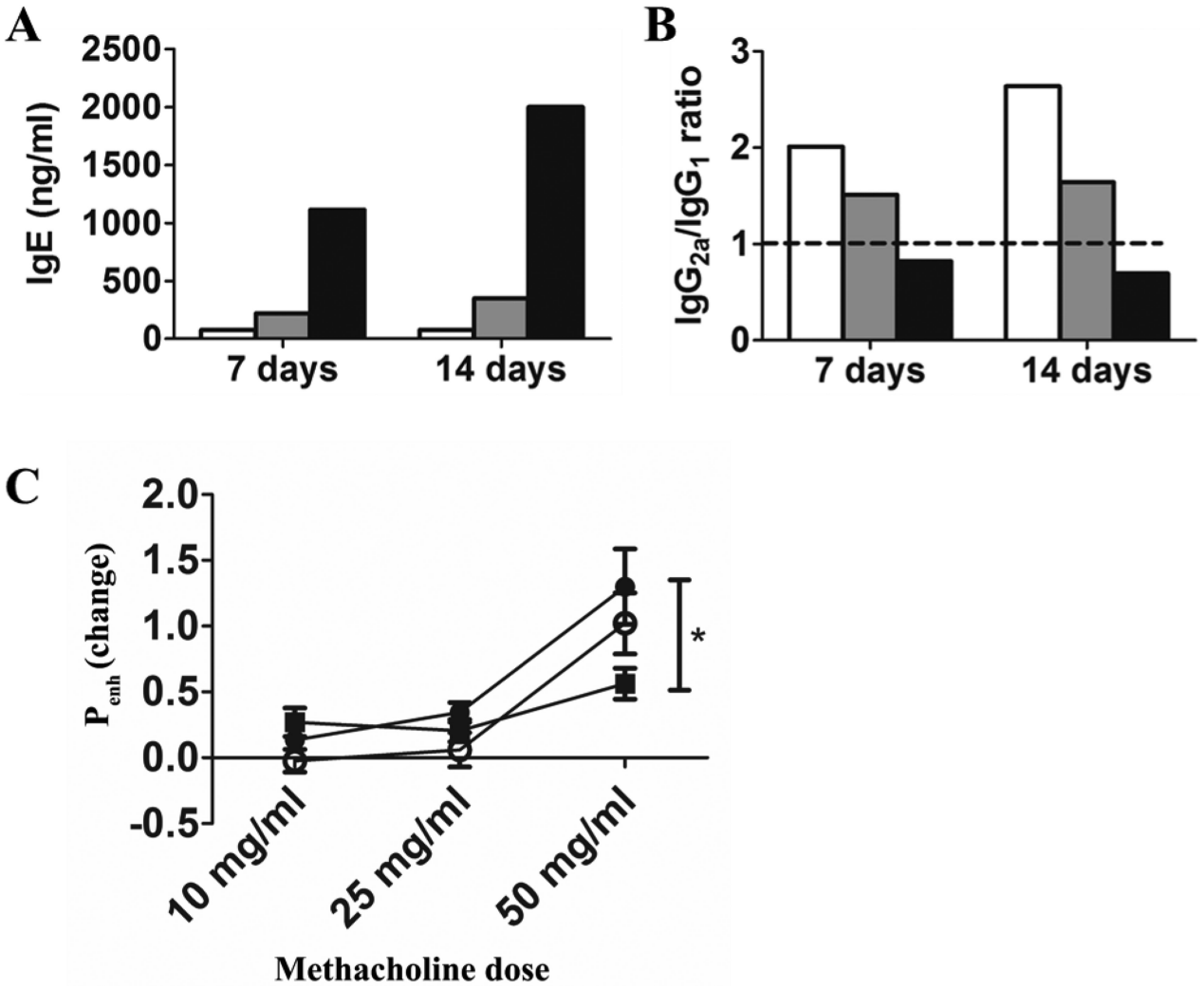

Figure 5. Serum antibody responses and airway hyperreactivity in response to repeated inhalation of *A. fumigatus* spores

A. Pooled serum (n=3 mice; duplicate samples) IgE concentrations (ng/ml) determined by ELISA. Exposure groups - (white bars) Control, (grey bars) 10^4 spores, (black bars) 10^5 spores. **B.** Ratio of serum IgG_{2a}/IgG_1. Exposure groups - (white bars) Control, (grey bars) 10^4 spores, (black bars) 10^5 spores and (n=3 mice; duplicate samples). **C.** AHR analysis to varying concentrations (10, 25 and 50 mg/ml) of methacholine at 10 days post final exposure. Dose-dependent significant increase in AHR observed in mice repeatedly dosed to 10^5 *A. fumigatus* spores and challenged with 50 mg/ml methacholine (n=3 mice). Exposure groups - (●) Control, (○) 10^4 spores, (▼) 10^5 spores.